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# Directed growth of fibroblasts into three dimensional micropatterned geometries via self-assembling scaffolds

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ABSTRACT

We describe the use of conventional photolithography to construct three dimensional (3D) thin film scaffolds and direct the growth of fibroblasts into three distinct and anatomically relevant geometries: cylinders, spirals and bi-directionally folded sheets. The scaffolds were micropatterned as two dimensional sheets which then spontaneously assembled into specific geometries upon release from the underlying substrate. The viability of fibroblasts cultured on these self-assembling scaffolds was verified using fluorescence microscopy; cell morphology and spreading were studied using scanning electron microscopy. We demonstrate control over scaffold size, radius of curvature and folding pitch, thereby enabling an attractive approach for investigating the effects of these 3D geometric factors on cell behaviour.



## 1. Introduction

Cells cultured in three dimensional (3D) microenvironments often exhibit physiological differences from those cultured on flat substrates [1,2]. Tumor models, for example, have shown markedly improved efficacy when cultured in 3D systems that promote the organization of cells into microstructures observed *in vivo* [3–5]. In order to explore the effect that 3D environments have on cell behaviour, a variety of 3D scaffolds have been utilized including porous hydrogels [6], electrospun fiber meshes and rigid polymeric substrates [7]. One limitation shared by these scaffolds is their inherently random spatial orientation. Solid free-form fabrication methods have been used to overcome this limitation by enabling precise control over 3D scaffold features such as pore size and connectivity [8]. Although these methods have provided new capabilities for the study of cells in 3D, they are serial processes which often require specialized equipment that is not easily accessible. Additionally, these processes have limited utility in cost-effective fabrication of curved and simultaneously patterned structures.

Lithographic methodologies including electron beam lithography, soft-lithography, and photolithography have increasingly been utilized to precisely pattern materials for cell culture [9–11]. For example, soft-lithography has been used to functionalize two-dimensional (2D) surfaces and subsequently study cell attachment [12], cell–matrix [13] interactions, as well as the influence of cell geometry on their fate [14]. Cell morphology and adhesion have also been modulated by lithographically patterning materials of varying stiffness [15] and micro- and nano-topographical features [16,17]. Recently, lithographic approaches have also been developed to create 3D patterned cell substrates such as micropatterned hydrogels [18]. By encapsulating cells in photopatternable hydrogels, multilayered cell culture geometries with well-defined micron scale features such as channels for improved nutrient diffusion are feasible [19,20]. Still, curved geometries such as cylinders, spirals and folded sheets are difficult to construct and pattern using these layer-by-layer lithographic methods. Multilayered cylindrical scaffolds have been demonstrated [21,22], but these scaffolds were rolled-up manually.

In this paper, we adapted a self-assembly strategy [23,24] to construct 3D thin film cell scaffolds and directed the growth of fibroblasts into three geometries that are reminiscent of microstructures abundant in tissues within the body: cylinders (e.g. vasculature, ducts), spirals (glandular coils, cochlea), and bi-directionally folded sheets (gyri/sulci, intestinal villi). An overview of our 3D cell culturing strategy is illustrated in Fig. 1. Briefly, micropatterned scaffolds were fabricated on 2D silicon (Si)

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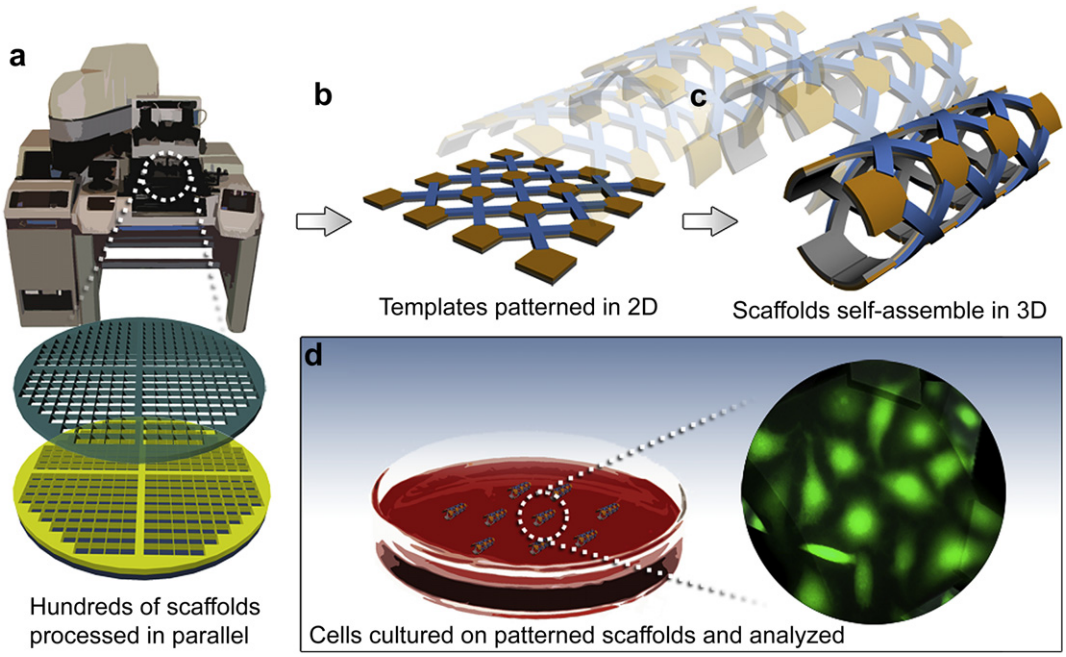


**Fig. 1.** Schematic diagram illustrating the 3D cell culture strategy. (a) Conventional photolithography was used to fabricate scaffolds *en masse*. (b) Scaffolds were initially micropatterned as 2D templates. (c) Scaffolds self-assembled from 2D to 3D geometries on release from the Si substrate. (d) Cells were cultured on the scaffolds and later analyzed using standard microscopy techniques.

substrates using conventional photolithography (Fig. 1a). Hundreds of 4 mm square 2D templates with well-defined sizes, shapes, and materials could be simultaneously fabricated on a single 3 inch diameter Si wafer (Fig. 1b). Upon dissolving the underlying sacrificial layer (Fig. 1c), the scaffolds spontaneously folded into their final curved geometries due to the differential in stresses across multilayer flexible hinges. The scaffolds were then rinsed, coated with the extracellular matrix protein fibronectin to promote cell attachment, and used to guide the 3D growth of fibroblasts (Fig. 1d). The highlight of our technique is that it harnesses the strengths of 2D lithographic patterning while enabling the fabrication of curved, complex 3D cell scaffolds.

## 2. Materials and methods

### 2.1. Chemicals and materials

#### 2.1.1. Cell culture

L929 mouse fibroblast cells were purchased from ATCC (cat # CCL-1). The following chemicals were purchased from Sigma–Aldrich (Sigma): Minimum Essential Medium Eagle, horse serum, sodium pyruvate, nonessential amino acids and fibronectin from bovine plasma. Phosphate-buffered saline (PBS) was purchased from Gibco.

#### 2.1.2. Fluorescent microscopy

Calcein AM was purchased from both Sigma and Invitrogen. Ethidium homodimer-1 was purchased from Invitrogen.

#### 2.1.3. Fabrication

Si wafers were purchased from Montco Silicon Technologies. Gold (Au) and copper (Cu) slugs were purchased from Alfa Aesar, chromium (Cr) rods from Fil-Tech Inc, Au plating solution from Technic Inc, and the following chemicals from MicroChem Corp.: SC 1800 series photoresist (PR), Microposit 351 Developer, SU-8 2015 photoresist (SU-8), and SU-8 developer. Iron(III) chloride was purchased from Sigma. Acetone, 2-propanol, and ethanol solvents were purchased from J.T. Baker and Fisher Chemicals.

#### 2.1.4. Scanning electron microscopy

Sodium cacodylate, formaldehyde, glutaraldehyde, and uranyl acetate were purchased from Electron Microscopy Sciences. The following chemicals were purchased from J.T. Baker: sucrose, sodium hydroxide, sodium acetate anhydrous, hydrochloric acid, and osmium tetroxide. Calcium chloride was purchased from Fisher Chemicals and sodium barbital from Sigma.

### 2.2. Fabrication of the scaffolds

The fabrication process is an adaptation of that used in prior work [23,24] specifically designed to include the bio-inert materials Au and SU-8 for cell culture. The details of the fabrication process are as follows: Si wafer substrates were first rinsed with acetone, 2-propanol, and distilled water and were then dried under nitrogen gas. A 15 nm Cr adhesion layer followed by a 150 nm Cu sacrificial layer was thermally evaporated on the wafer (Fig. 2a) at a pressure of approximately $10^{-5}$ Torr. Next, a lift-off metallization step was used to precisely pattern the stress driving layer that was composed of Cr and Au. Briefly, a 2.7 μm layer of PR was spin-coated at 3000 rpm and baked for 1 min at 115 °C on a hot plate. The micrometer features were patterned by selectively exposing the Si wafer to ultraviolet light through a photomask that was designed using AutoCAD and laser printed on plastic film at 40,640 dots per inch by Fineline Imaging, Inc. The wafer was developed in Microposit 351 Developer resulting in regions of bare Cu and regions of PR. For structures with a single direction of curvature, a stressed bilayer of 140 nm Cr and 30 nm Au was thermally evaporated as before, and upon removing the PR in acetone, only the patterned bilayer remained (Fig. 2b). The lithography step was repeated in order to electrodeposit 270 nm of Au. A similar process was used to deposit 11.5/245/59 nm of Cr/Au/Cr which is necessary for the bi-directionally folded structures. Once the Cr/Au bilayer or Cr/Au/Cr trilayer was complete, we patterned the areas that would become rigid panels in the folded scaffolds. This was achieved by either electrodepositing a 3 μm thick layer of Au or photopatterning a 10 μm thick layer of SU-8 (Fig. 2c). Finally, PR was photopatterned atop the flexible hinge regions (Fig. 2d) and the 2D templates were released by dissolution of the Cu sacrificial layer in a 40 wt% aqueous solution of iron(III) chloride with 5 wt% hydrochloric acid (Fig. 2e). On dissolution at 40 °C, the templates lifted-off and spontaneously assembled into 3D scaffold geometries in less than 1 min (Fig. 2f). They were rinsed multiple times in deionized water and stored in 1× PBS, pH 7.4, for cell culture.

### 2.3. Preparation of substrates for cell culture

Scaffolds were transferred from PBS at room temperature to chilled (4 °C) PBS containing 25 μg/mL bovine fibronectin. They were incubated for at least 1.5 h in a bio-safety hood at room temperature before being rinsed with warm 1× PBS, pH 7.4, followed by one rinse with warm cell culture medium as specified in the following section.

### 2.4. Cell culture

L929 mouse fibroblast cells were cultured in 75 $cm^2$ culture flasks containing 85% Minimum Essential Medium Eagle with L-glutamine, sodium bicarbonate, 10%